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Report

Actin Dynamics Affect Mitochondrial Quality Control and Aging in Budding Yeast

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Summary

Actin cables of budding yeast are bundles of F-actin that extend from the bud tip or neck to the mother cell tip, serve as tracks for bidirectional cargo transport, and undergo continuous movement from buds toward mother cells [1]. This movement, retrograde actin cable flow (RACF), is similar to retrograde actin flow in lamellipodia, growth cones, immunological synapses, dendritic spines, and filopodia [2–5]. In all cases, actin flow is driven by the push of actin polymerization and assembly at the cell cortex, and myosin-driven pulling forces deeper within the cell [6–10]. Therefore, for movement and inheritance from mothers to buds, mitochondria must "swim upstream" against the opposing force of RACF [11]. We find that increasing RACF rates results in increased fitness of mitochondria inherited by buds and that the increase in mitochondrial fitness leads to extended replicative lifespan and increased cellular healthspan. The sirtuin *SIR2* is required for normal RACF and mitochondrial fitness, and increasing RACF rates in *sir2Δ* cells increases mitochondrial fitness and cellular healthspan but does not affect replicative lifespan. These studies support the model that RACF serves as a filter for segregation of fit from less-fit mitochondria during inheritance, which controls cellular lifespan and healthspan. They also support a role for Sir2p in these processes.

Results and Discussion

Altering the Rate of Retrograde Actin Cable Flow Affects Mitochondrial Quality Control during Inheritance

Microtubules and microfilaments are well known as tracks for intracellular organelle and cargo movement. In many cases, the tracks are static. However, in the budding yeast, the tracks are moving in the direction that is opposite that of organelles as they move from mother cells to buds during cell division. Here, we tested the hypothesis that retrograde actin cable flow (RACF) can exercise mitochondrial quality control during cell division. If RACF serves as a filter to prevent low-functioning mitochondria from moving from mother cells to buds, then increasing the rate of RACF should result in inheritance of fitter, more-motile mitochondria, and slowing the rate of RACF should have the opposite effect.

To test this hypothesis, we measured mitochondrial motility and function in *myo1Δ* and *tpm2Δ* yeast, which show altered retrograde cable flow rates. Myo1p, a type II myosin that localizes to the bud neck, generates pulling forces for RACF and for contractile ring closure [9]. Tpm2p is one of two tropomyosins in yeast. The only known function of Tpm2p is to regulate RACF by regulating the binding of Myo1p to actin cables [9].

Deletion of *MYO1* or *TPM2* has no obvious effect on actin cable abundance, polarization of the actin cytoskeleton (as assessed by enrichment of actin patches, endosomes that are invested with a coat of F-actin, in the bud), or the steady-state level of the sirtuin Sir2p (Figure 1A and Figure S1 available online). Deletion of *MYO1* results in 28.5% and 21.4% decreases in the velocity of RACF and retrograde mitochondrial movement, respectively. Conversely, deletion of *TPM2* results in 32.3% and 28.1% increases in the velocities of retrograde actin cable and mitochondrial movement, respectively (Figures 1B–1E). Thus, altering the rate of RACF results in a corresponding change in the velocity of retrograde mitochondrial movement.

Next, we measured the velocity of anterograde, bud-directed mitochondrial movement. Since mitochondria undergoing anterograde movement are moving against the opposing force of RACF, the actual velocity of anterograde mitochondrial movement, in the frame of reference of the actin cable, is significantly greater than the apparent velocity of mitochondrial movement, in the frame of reference of the cell. To account for these directional force considerations, we calculated an adjusted velocity of anterograde mitochondrial movement, which better reflects the intrinsic velocity of anterograde movement, by subtracting the effect of RACF (Figure S1).

The measured velocities of anterograde mitochondrial movement were not different between the mutants. However, taking into account the opposing force of RACF, the adjusted real rates are significantly different. We find that increasing RACF rates by deletion of *TPM2* results in a 29.9% increase in the adjusted velocity of anterograde mitochondrial movement. Moreover, decreasing the rate of RACF by deletion of *MYO1* reduces the adjusted mitochondrial anterograde velocity by 13.9% (Figures 1F, 1G, and S1). Motility reflects the ability to assemble functional motility proteins and provide energy to the motility apparatus and, thus, is one indicator of the fitness of an organelle. Our finding that an increase in RACF rates results in increased anterograde mitochondrial motility supports the model that RACF exercises mitochondrial quality control during inheritance.

Consistent with this, we find that mitochondrial redox state, measured using mitochondria-targeted redox-sensing GFP1 (mito-roGFP1), correlates with rates of RACF and anterograde mitochondrial motility. roGFP contains two surface-exposed cysteines. Oxidation or reduction of these cysteines occurs in response to the redox state of the environment and alters the excitation spectrum of roGFP [12]. roGFP has been targeted to yeast mitochondria where it serves as an effective biosensor for mitochondrial redox state in living cells [13]. Indeed, in yeast, mito-roGFP fluorescence ratios show a linear dose-response relationship upon treatment with oxidizing or

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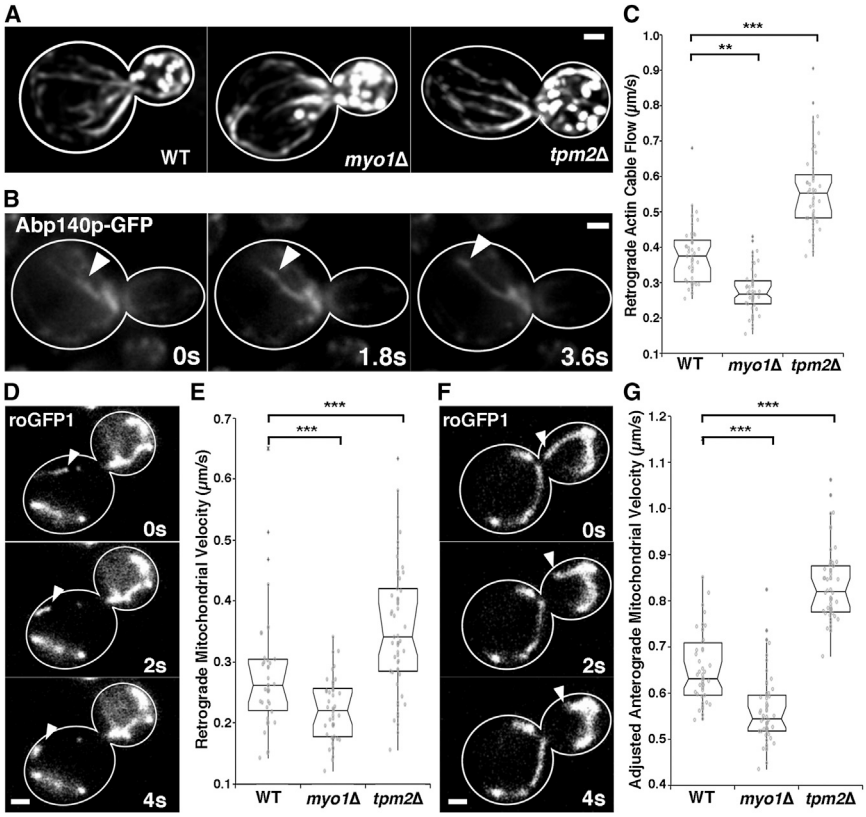

Figure 1. RACF Affects Mitochondrial Motility, but Does Not Alter Actin Cable Abundance or Polarity

(A) Images present rhodamine-phalloidin-stained actin in WT, *myo1Δ*, and *tpm2Δ* cells.

(B) Still frames of a time-lapse series show Abp140p-GFP-labeled actin cables undergoing retrograde flow. Arrowheads indicate a fiduciary mark on a motile actin cable.

(C) Notched-box dot plot shows the velocity of RACF in WT, *myo1*Δ, and *tpm2*Δ yeast. The central band in the box represents the median, and boxes indicate the middle quartiles; whiskers extend to the 5th and 95th percentiles, and red points indicate outliers (defined as quartile 1–1.5× interquartile range and quartile 3 + 1.5× interquartile range) (n = 50 from three trials).

(D) Still frames from a time-lapse series illustrate retrograde mitochondrial movement in WT cells expressing mito-roGFP.

(E) Notched-box dot plot presents the velocity of retrograde mitochondrial movement in WT, *myo1*Δ, and *tpm2*Δ yeast (n = 40–60 from three trials).

(F) Still frames from a time-lapse series show anterograde mitochondrial movement in WT cells expressing mito-roGFP1.

(G) Notched-box dot plot presents the adjusted velocity of adjusted anterograde mitochondrial movement in WT, *myo1Δ*, and *tpm2Δ* yeast (n = 40–60 from three trials).

**p < 0.01 and ***p < 0.001. The p values were calculated using Kruskal-Wallis testing. Scale bars represent 1 μm. Cell outlines are shown in white. See also Figure S1.

reducing agents [14]. Moreover, treatment with saturating levels of a membrane-permeable reductant or oxidant results in a 40% increase or a 19% decrease, respectively, in mito-roGFP redox ratio, compared to untreated cells (Figure S2). Thus, a higher ratio indicates a more reducing environment. We find that increasing the rate of RACF by deletion of *TPM2* increases mitochondrial redox ratio by 19.7% and that reduction of the rate of RACF by deletion of *MYO1* renders mitochondria 11.7% more oxidizing (Figures 2A and 2B). Thus, by two different measures, motility and redox state, cells with increased RACF rates have higher functioning mitochondria.

Yet to be determined is why mitochondria in *tpm2Δ* and *myo1Δ* cells are more or less reducing and motile, respectively, compared to mitochondria in wild-type (WT) cells. Previous studies revealed that mitochondria with higher membrane potential, aconitase activity, redox state, and ROS are preferentially inherited by daughter cells during yeast cell division [13, 15, 16]. This segregation of functional from less-functional mitochondria is mediated, in part, by anchorage and retention of newly inherited mitochondria in the developing daughter cell. It is also required for mother-daughter age asymmetry, the process whereby mother cells continue to age, and daughter cells are, for the most part, born young [13].

To determine whether the changes in mitochondrial redox state and motility in *tpm2Δ* and *myo1Δ* cells are due to RACF effects on mitochondrial quality control during inheritance, we assessed the effect of modulating RACF rate on mitochondrial redox states in mother and daughter cells. In WT cells, there is a small, but statistically significant, asymmetry in mitochondrial redox state between mother and bud. *tpm2Δ* cells show a 28% larger difference between mother and bud. Conversely, functional segregation of mitochondria is not detectable in *myo1Δ* cells, which have decreased RACF rates (Figure 2C). Thus, segregation of more reducing and motile mitochondria from those that are less reducing and motile among mother cells and buds is enhanced by deletion of *TPM2* and the associated increase in RACF rate. This is the first direct evidence that RACF serves as a filter to prevent inheritance of less-motile and more-oxidized mitochondria and to promote inheritance of more-motile and reduced mitochondria during yeast cell division.

Altering the Rate of RACF Affects Cellular Lifespan and Healthspan through Effects on Mitochondrial Function

Recent studies indicate that altering the function of mitochondria in daughter cells can affect yeast lifespan. Specifically, yeast that inherit mitochondria that are more reducing and have lower reactive oxygen species (ROS) have extended lifespan, while yeast that inherit mitochondria that are more oxidized and have more ROS have shortened lifespans [13, 17]. Therefore, we tested the effect of increasing the rate of RACF, and the associated increase in mitochondrial motility and redox state, on yeast lifespan and healthspan.

Aging studies in yeast can model two distinct forms of cellular aging. Chronological lifespan, the survival time of stationary-phase, nondividing yeast cells, is a model for stress resistance in postmitotic cells. Replicative lifespan (RLS), the number of times that a cell can divide prior to senescence, is a model for aging of division-competent cells. The mean RLSs of WT, *tpm2Δ*, and *myo1Δ* cells are 22.0 ± 1.0, 27.0 ± 1.4, and 17.2 ± 0.9 generations, respectively (Figure 2D). Thus, deletion of *TPM2*, which results in an increase in

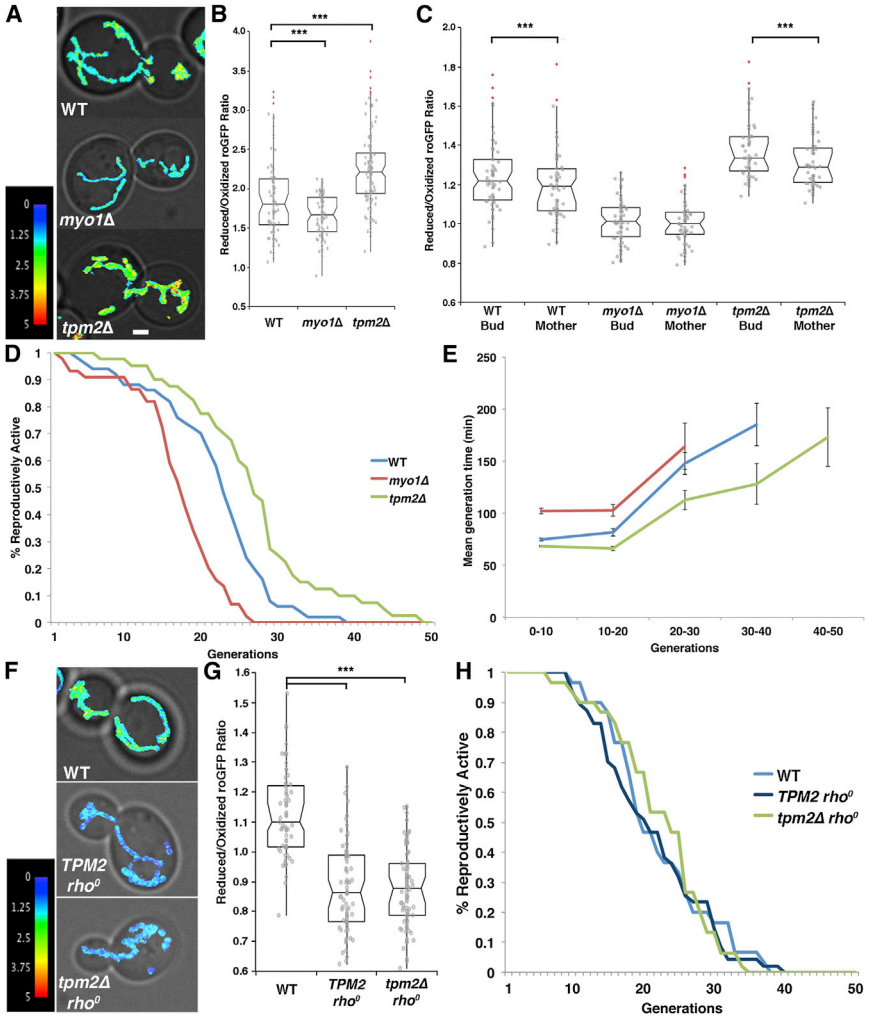

Figure 2. Altering RACF Changes Quality and Asymmetric Inheritance of Mitochondria, which in Turn Affects Cell Lifespan and Healthspan

(A) Mito-roGFP1 was used to visualize the redox state of mitochondria in WT, *myo1Δ*, and *tpm2Δ* cells. Images are reduced:oxidized mito-roGFP1 ratios overlaid on phase images. Color scale indicates ratio values; higher numbers and warmer colors indicate more-reducing mitochondria. Scale bar represents 1 μm.

(B) Notched-box dot plot shows the average reduced:oxidized mito-roGFP1 ratio in WT, *myo1Δ*, and *tpm2Δ* cells (n = 97–143 cells per strain). Data are representative of three experiments. ***p < 0.001, using the nonparametric Kruskal-Wallis test.

(C) Quantitation of asymmetric segregation of fit from less-fit mitochondria between mother and daughter cells is presented. The reduced:oxidized mito-roGFP1 ratio was measured in mother cells and buds in cells containing large buds (bud diameter >50% the diameter of the mother cell) (n = 55–65 cells per strain). Data are representative of two trials. ***p < 0.001, calculated by Wilcoxon paired difference test.

(D) RLSs of WT, *myo1Δ*, and *tpm2Δ* cells were determined as described in the Supplemental Experimental Procedures. Mann-Whitney U test was performed to determine p values: WT versus *tpm2Δ*, 0.004; and WT versus *myo1Δ*, 0.001.

(E) Mean generation time was determined during the RLS assay by recording the time between the emergence of consecutive buds from the same mother. Error bars represent SEM in (D) and (E) (n = 40–50 cells per strain). Data shown are representative of three trials.

(F) *rho*^{0} cells were produced by ethidium bromide treatment as described in the Supplemental Experimental Procedures. Images are representative reduced:oxidized roGFP ratios for WT (top), *TPM2 rho*^{0} (middle), and *tpm2Δ rho*^{0} (bottom) cells.

(G) Notched-box dot plot shows the average reduced:oxidized mito-roGFP1 ratio in WT, *TPM2 rho*^{0}, and *tpm2Δ rho*^{0} cells (n = 63–83 cells per strain). Data are representative of three experiments. ***p < 0.001, using nonparametric Kruskal-Wallis testing.

(H) RLSs of WT, *TPM2 rho*^{0}, and *tpm2Δ rho*^{0} cells are shown (n = 30–50 cells per strain). Data are representative of two experiments. Mann-Whitney U statistical analysis reveals no significant difference among all three groups.

See also Figure S2.

retrograde flow rate, extends RLS by 23% compared to WT cells. Conversely, deletion of *MYO1* results in a decrease in RLS by 22% compared to WT cells. Since Myo1p also functions in contractile ring closure, it is possible that the decreased RLS may be due to Myo1p function in processes other than RACF. However, the only known function of Tpm2p is to control RACF. Therefore, we conclude that increasing RACF can extend lifespan in yeast.

The healthspan of an organism is the period during which it is generally healthy and free of disease or age-related symptoms. The mean generation time of yeast increases as they age [13, 18]. Therefore, maintaining a short generation time is an indicator of cellular healthspan in yeast. *tpm2Δ* cells, which have increased RACF rates and mitochondria that are more motile and reducing, have a shorter generation time overall and also maintain a short generation time for more generations than WT cells. Decreasing the rate of RACF has the opposite effect (Figure 2E). Thus, changes in cytoskeletal dynamics can affect mitochondrial quality control during inheritance and alter RLS and cellular healthspan in a manner that correlates with mitochondrial fitness.

To determine whether the extended RLS that occurs upon deletion of *TPM2* is due to effects on mitochondrial function, we studied the effect of deletion of *TPM2* on lifespan in a rho^0 strain, which has no mitochondrial DNA (mtDNA). Loss of mtDNA does not affect actin cable abundance or actin patch polarity in cells that contain *TPM2* and in *tpm2Δ* cells (Figure S2). However, since mtDNA encodes mitochondrial respiratory chain components, rho^0 cells cannot grow in media containing a nonfermentable carbon (glycerol) as a sole carbon source (Figure S2). Consistent with this, our mito-roGFP measurements indicate that mitochondria in rho^0 cells are 22% more oxidized compared to mitochondria in rho$^+$ cells, both in cells that contain *TPM2* and in *tpm2Δ* cells. Interestingly, the redox state of mitochondria in rho^0 *TPM2* cells is not significantly different from that of rho^0 *tpm2Δ* cells (Figures 2F and 2G). Thus, the increase in mitochondrial redox state that occurs upon deletion of *TPM2* requires mtDNA.

If extension of lifespan in *tpm2Δ* cells is due to increased mitochondrial function, then the loss of mitochondrial respiratory activity and respiration-dependent processes that occurs upon deletion of mtDNA should prevent lifespan extension.

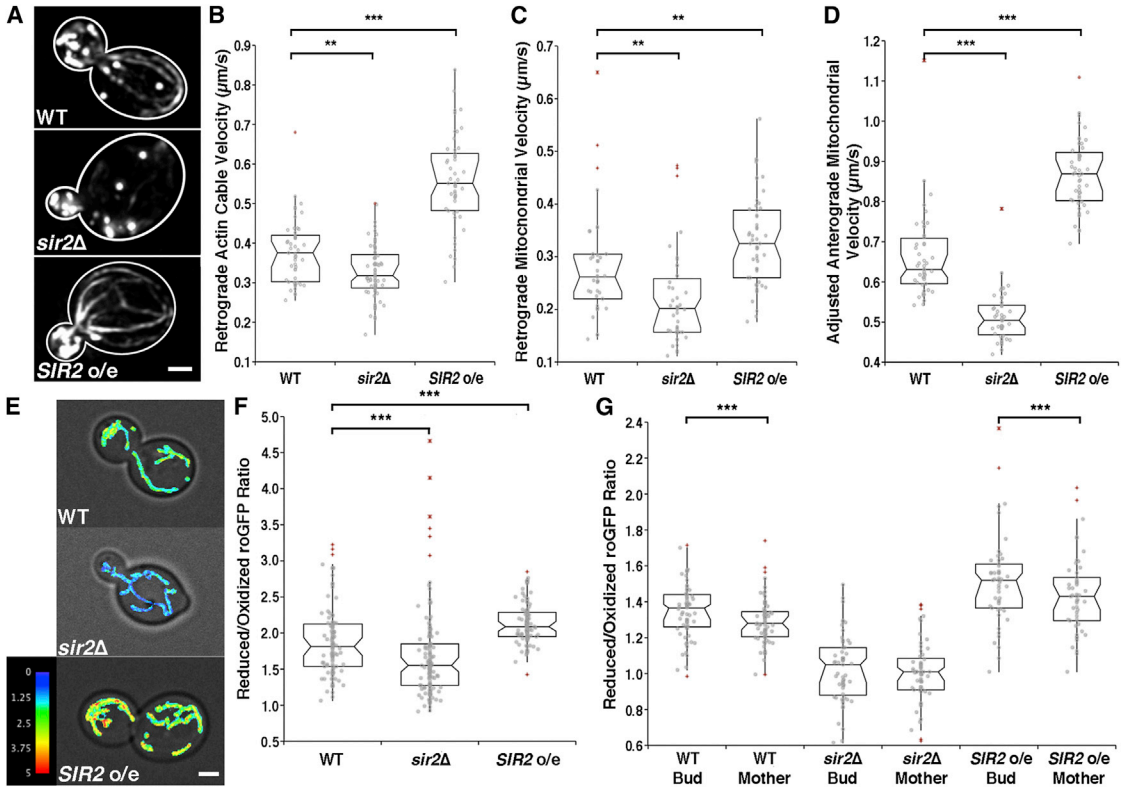

Figure 3. Sir2p Regulates Mitochondrial Inheritance, Fitness, Motility, and RACF

(A) Images present rhodamine-phalloidin-stained actin in WT, *sir2Δ*, and cells overexpressing *SIR2* (*SIR2* o/e).

(B–D) Quantitation of RACF rates (B), velocities of retrograde mitochondrial movement (C), and adjusted velocities of anterograde mitochondrial movement (D) in WT, *sir2Δ*, and *SIR2* o/e cells is shown (n = 40–69 cells per strain). Data are pooled from three trials.

(E) Images show reduced:oxidized mito-roGFP1 ratios overlaid on phase images in WT, *sir2Δ*, and *SIR2* o/e cells. Color scale indicates ratio values; higher numbers and warmer colors indicate more-reducing mitochondria (n = 97–127 cells per strain). Data are representative of three trials.

(F) Quantitation of average reduced:oxidized mito-roGFP1 ratio within individual cells is shown (n = 50–66 cells per strain). Data are representative of three trials. Higher values indicate more-reducing mitochondria.

(G) The average reduced:oxidized mito-roGFP1 ratio was determined in mother cells and buds as for Figure 2.

**p < 0.01 and ***p < 0.001. The p values were calculated using the nonparametric Kruskal-Wallis test for (B)–(F) and the nonparametric Wilcoxon paired difference test for (G). Scale bars represent 1 μm. See also Figure S3.

Deletion of mtDNA can affect RLS in some genetic backgrounds. We confirmed that deletion of mtDNA does not affect RLS in BY4741 cells: the average RLS of rho$^+$ cells (22.2 ± 1.34) is similar to that of rho^0 cells (21.4 ± 1.2). More importantly, deletion of *TPM2* in rho^0 cells does not affect RLS (21.4 ± 1.2 in rho^0 versus 22.7 ± 1.29 in *tpm2Δ* rho^0 cells) (Figure 2H). Thus, mitochondrial function is required for the RLS extension that occurs upon deletion of *TPM2* and the associated increase in RACF.

Sir2p Affects RACF and Mitochondrial Quality Control

Previous studies indicate that deletion of Sir2p alters yeast actin: it results in decreased actin cable abundance, increased sensitivity of yeast to the growth-inhibiting effects of Latrunculin-A (an agent that binds to G-actin and results in rapid actin disassembly), and reduces levels of natively folded actin protein [19]. We confirmed that deletion of *SIR2* reduces the number of actin cables and results in depolarization of actin patches. Deletion of *SIR2* also results in a decrease in the velocities of RACF and retrograde mitochondrial movement. Conversely, mild (2.2-fold) overexpression of fully functional, HA-tagged *SIR2* has the opposite effect (Figures 3A–3C and S3). Thus, our studies reveal a novel role for Sir2p in the control of RACF. The levels of properly folded actin are reduced in *sir2Δ* cells and *SIR2* has genetic interactions with the formin (Bni1p) that nucleates actin for actin cable assembly and movement in the bud tip [19]. Therefore, it is possible that the reduced amount of substrate (native actin) available for actin nucleation and actin cable elongation in *sir2Δ* cells affects actin cable thickness, abundance, retrograde flow, and function in the establishment and maintenance of cell polarity.

Consistent with the observed effect on RACF, deletion of *SIR2* also results in a decrease in the adjusted velocity of anterograde mitochondrial motility, a more oxidizing mitochondrial redox environment, and defects in segregation of more reduced from more-oxidized mitochondria between mother and daughter cells. Mild overexpresssion of *SIR2* has the opposite effects (Figures 3D–3G and S3). Deletion of *SIR2* has more severe effects on adjusted anterograde mitochondrial movement compared to deletion of *MYO1*. The simplest explanation for this difference is that deletion of *SIR2* has general effects on actin cable abundance, thickness, and polarity, in addition to effects on RACF. Overall, these findings support a role for Sir2p in many aspects of the actin cytoskeleton, including RACF, and in mitochondrial quality control.

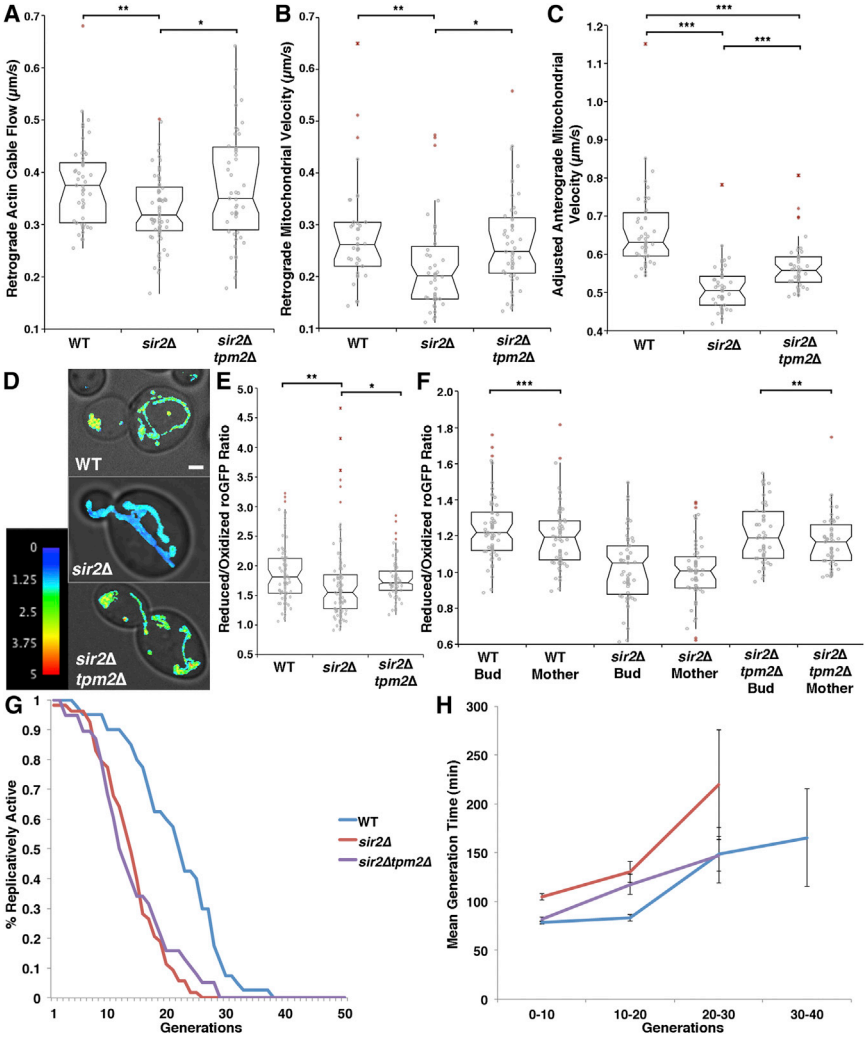

Figure 4. Modulation of RACF Rates Promotes Mitochondrial Motility and Cell Healthspan in *sir2Δ* Yeast

(A–C) Quantitation of RACF rates (A), velocities of retrograde mitochondrial movement (B), and adjusted velocities of anterograde mitochondrial movement (C) in WT, *sir2Δ*, and *sir2Δ tpm2Δ* cells are shown (n = 39–51 cells per strain). Data are pooled from three trials.

(D) Images show reduced:oxidized mito-roGFP1 ratio overlaid on phase images in WT, *sir2Δ*, and *sir2Δ tpm2Δ* cells. Color scale at right indicates ratio values; higher numbers and warmer colors indicate more-reducing mitochondria. Scale bar represents 1 μm.

(E) Quantitation of average reduced:oxidized mito-roGFP1 ratio within individual cells is presented (n = 60–100 cells per strain). Data shown are representative of two trials.

In (A)–(C) and (E), *p < 0.05, **p < 0.01, and ***p < 0.001 were calculated using the nonparametric Kruskal Wallis test.

(F) Quantification of asymmetric segregation of fit from less-fit mitochondria during cell division is shown. The average reduced:oxidized mito-roGFP1 ratio was measured in mother cells and buds (n = 50–53 cells per strain). Data shown are representative of two trials. **p < 0.01 and ***p < 0.001. The p values were calculated using a nonparametric Wilcoxon paired difference test.

(G) RLSs of WT, *sir2*Δ, and *sir2Δ tpm2Δ* cells are shown. Mann-Whitney U test was performed to determine p values: WT versus *sirΔ*, < 0.0001; WT versus *sir2Δ tpm2*Δ, < 0.0001; and *sir2*Δ versus *sir2Δ tpm2Δ*, 0.5.

(H) Mean generation time was determined during the RLS assay as for Figure 2. Error bars represent SEM (n = 38–52 cells per strain). Data are representative of two trials.

See also Figure S4.

Increasing the Rate of RACF in a *sir2Δ* Cell Increases Mitochondrial Motility and Redox State and Cellular Healthspan

Because *SIR2* has many functions, we tested whether changes in mitochondrial movement and redox state could be abrogated by altering retrograde flow rates through deletion of *MYO1 or TPM2*. Indeed, deletion of *MYO1* in a *SIR2*-overexpressing strain restores the adjusted velocity of anterograde mitochondrial motility and mitochondrial redox state, to WT levels (Figure S4). On the other hand, increasing RACF rate by deletion of *TPM2* in *sir2Δ* cells improves but does not completely restore mitochondrial redox state, cell division-linked segregation of more-reduced from more-oxidized mitochondria, and anterograde mitochondrial motility (Figures 4A–4F). Increasing RACF in *sir2Δ* also restores polarity of the actin cytoskeleton but does not restore actin cable abundance (Figure S4). These findings provide further support for the concept that Sir2p affects mitochondrial function by RACF-dependent and -independent mechanisms, including general effects on actin cable abundance.

Finally, we studied the effect of increasing RACF in a sir2Δ on lifespan and healthspan. The mean RLS of *sir2Δ tpm2Δ* cells (14.2 ± 0.72) is similar to that of *sir2Δ* cells (14.3 ± 1.08) (Figure 4G). Thus, increasing RACF and mitochondrial motility and redox state in *sir2Δ* cells does not increase RLS. This finding indicates that Sir2p affects lifespan through multiple mechanisms, beyond mitochondria and actin dynamics, which is consistent with previous reports [20]. On the other hand, the mean generation time of *sir2Δ tpm2Δ* yeast is shorter than that of *sir2Δ* cells in young cells that have undergone zero to ten replications (Figure 4H). Thus, increasing RACF rates can promote cellular healthspan in a *sir2Δ* cell, potentially through effects on mitochondrial redox state and motility and/or effects on actin cable polarity.

Here, we describe novel roles for the actin cytoskeleton in mitochondrial quality control and lifespan regulation. Specifically, our studies support the model that RACF exercises mitochondrial quality control by serving as a filter to prevent less-motile and more-oxidized mitochondria from leaving the mother cell and select for the inheritance of more-motile and -reducing mitochondria. Retrograde actin network flow also occurs at lamellipodia, growth cones, immunological synapses, dendritic spines, and filopodia of mammalian cells and can drive organelle and particle movement [2–5, 21]. Therefore, it is possible that similar quality control mechanisms exist in other eukaryotes, perhaps selecting subpopulations of organelles that will be delivered to cellular regions with a need for mitochondrial function.

We also find that modulation of actin cable dynamics can alter lifespan and healthspan in a manner that correlates with and is dependent upon mitochondrial function, and we reveal a new role for Sir2p, a conserved lifespan modulator, in RACF. Mitochondria are aging determinants in yeast and other cell types, and mutations in mitochondrial quality control during inheritance can affect lifespan in yeast [13]. However, mitochondria are not the only aging determinant whose segregation is influenced by retrograde actin flow. Oxidatively damaged proteins are also retained in the mother cell by mechanisms that are not fully understood but are dependent upon the actin cytoskeleton [19, 22]. Moreover, vacuoles, which are lysosome-like organelles that undergo actin cable-dependent movement in yeast, are also aging determinants: vacuolar acidity declines with replicative age, and preventing the decline in vacuolar acidity suppresses age-associated declines in mitochondrial function and extends lifespan [23]. Thus, it is possible that RACF affects asymmetric distribution of other aging determinants.

Supplemental Information

Supplemental Information includes Supplemental Experimental Procedures, four figures, and two tables and can be found with this article online at http://dx.doi.org/10.1016/j.cub.2013.10.022.

Acknowledgments

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